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TITLE: Evaluation of the immunologic impact of RAF Inhibitors to Guide Optimal Combination of RAF inhibitors and Immunotherapy for the treatment of Advanced Melanoma

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14. ABSTRACT During this first year of funding, we have made the following important observations. (1) T cells activated in vitro in the presence of BRAF inhibitors demonstrate a pattern of paradoxical activation characterized by upregulation of activation markers (CD69, PD-1, ICOS) and hyperactivation of the ERK signaling pathway. (2) T cells activated in vivo in the presence of BRAF inhibitors also demonstrate a pattern of paradoxical activation demonstrated by increased T cell expansion in vivo and hyperactivation of the ERK signaling pathway. (3) T cells activated in vitro in the presence of MEK inhibitors demonstrate inhibited upregulation of activation markers (CD69, PD-1, ICOS) and inhibition of the ERK signaling pathway. (4) T cells activated in vivo in the presence of MEK inhibitors also demonstrate a pattern of diminished activation demonstrated by lower T cell expansion in vivo and inhibition of the ERK signaling pathway. These first two observations have been reported in a manuscript that has been accepted for publication in the Journal Cancer Immunology Research. In addition, we have completed the following milestones that will form the foundation for future work: (1) regulatory approval for mouse studies, (2) regulatory approval for human correlative studies and 3) expansion of the BRAF/PTEN mouse colony.					
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## Table of Contents

	<u>Page</u>
Coverletter.....	1
SF298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4-6
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusion.....	9
References.....	10

## INTRODUCTION:

Our hypothesis is that combination therapy with MAPK inhibitors and immunotherapy will result in more rapid and durable control of melanoma than either modality alone. This topic continues to be one of active interest with several recent publications adding to the knowledge in this area.<sup>1-9</sup> Thus far, this grant has supported work that resulted in the following publication :

Callahan, et al. Paradoxical activation of T cells via augmented ERK signaling mediated by a RAF inhibitor. **Cancer Immunology Research**. 2(1): 70-9. 2014.<sup>10</sup>

In addition, a second manuscript is in the early stages of development, focusing of the effects of MEK inhibitors on T cell function.

In the past year, we have made progress in the following areas:

**(1) Test the impact of targeted inhibitors (PLX4720, AZD6244) on T cell activation *in vitro* and *in vivo*.**

- a. Test the effect of targeted inhibitors on expression of clinically relevant markers (ICOS, CTLA-4, PD-1, Ki67) in T cells activated *in vitro*.
- b. Evaluate the effect of targeted inhibitors on activation and expansion of tumor-antigen specific transgenic T cells *in vivo*.

**(2) Characterize the effect of targeted inhibitors on the anti-tumor activity of checkpoint blockade (CTLA-4, PD-1) in an immunocompetant spontaneous mouse model of BRAF mutant melanoma.**

- a. Combine targeted inhibitors with CTLA-4 blockade (or PD-1 blockade) and evaluate therapeutic efficacy and immune markers in the tumor and periphery.
- b. Compare three schedules (concurrent/sequential/alternating) combining targeted inhibitors with checkpoint blockade.

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**1a) Test the effect of targeted inhibitors on expression of clinically relevant markers (ICOS, CTLA-4, PD-1, ki67) in T cells activated *in vitro*.**

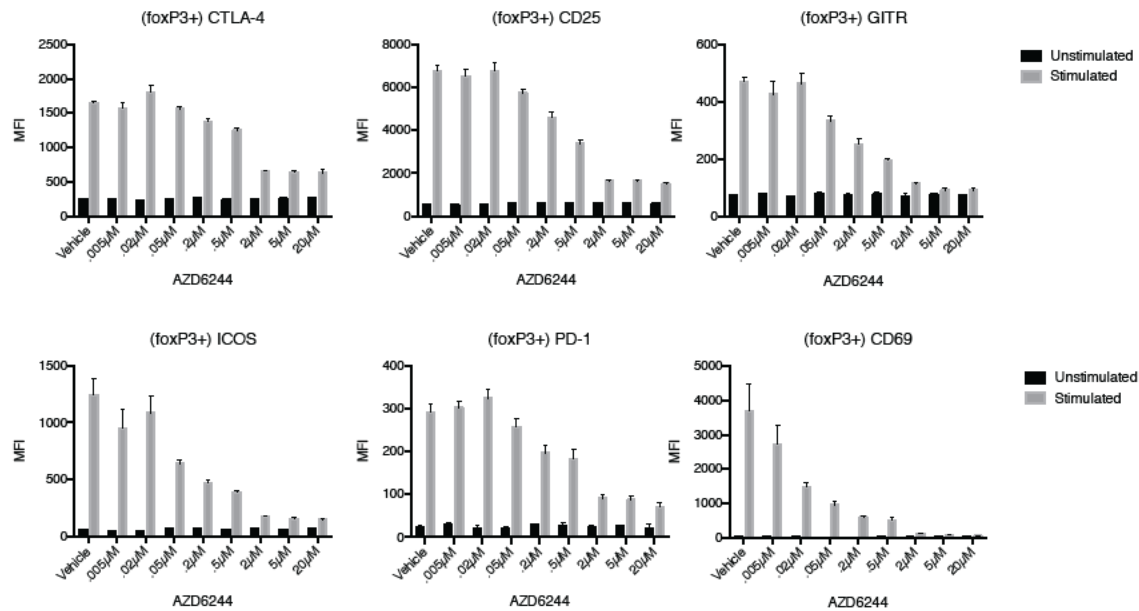
This first aim was initially explored primarily for the BRAF inhibitors (as described in prior annual report) and primarily in cultured murine T cells or human cell lines. In the past 1 year, we have expanded this analysis to include a detailed look at MEK inhibitors (specifically AZD6244) on T cell activation and expanded our analysis to the culture of primary human CD4 and CD8 T cells.

This analysis has included the following groups:

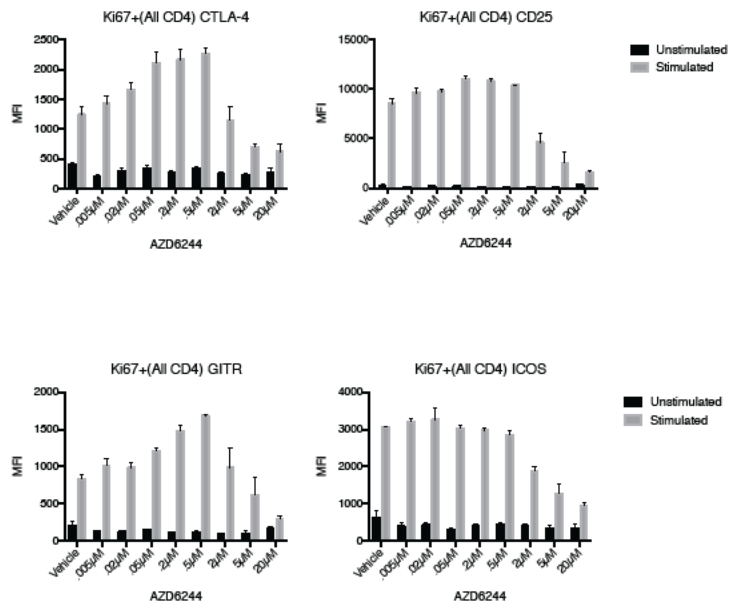
Analysis of BRAFi XL281 in mouse T cells, Jurkat cells, and human T cells.  
Analysis of BRAFi PLX4720 in mouse T cells, Jurkat cells, and human T cells.  
Analysis of MEKi AZD6244 in mouse T cells, Jurkat cells, and human T cells.

The following representative data depicts our approach to this analysis and reflects some of the more interesting findings to emerge from this analysis.

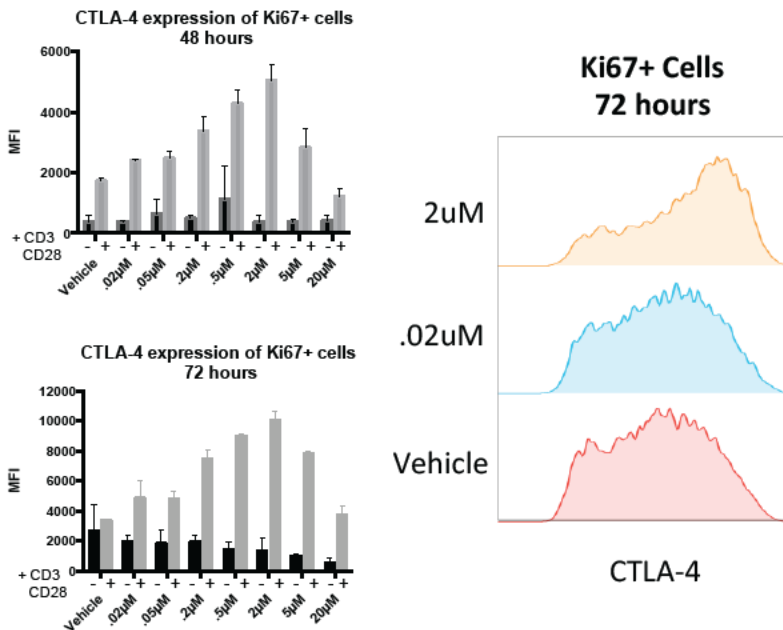




**Figure 3: AZD6244 effects Human Foxp3+ CD4+ (Regulatory) T cell activation in a concentration dependent manner.** Purified human CD4+ T cells were activated with a combination of anti-CD3 and anti-CD28 antibody in the presence of titrated concentrations of AZD6244 ranging from .005μM to 20μM. Flow analysis for indicated (CTLA-4, ICOS, CD25, ICOS, PD-1, CD69) markers was performed on all FoxP3+, CD4+ T cells.



**Figure 4: Phenotypic changes in Ki67+CD4+ cells treated with AZD6244,** Purified human CD4+ T cells were activated with a combination of anti-CD3 and anti-CD28 antibody in the presence of titrated concentrations of AZD6244 ranging from .005μM to 20μM. Flow analysis for indicated (CTLA-4, ICOS, CD25, ICOS) markers was performed on all ki67+, CD4+ T cells.



**Figure 5: Phenotypic changes in Ki67+CD4+ cells treated with AZD6244,** Purified human CD4+ T cells were activated with a combination of anti-CD3 and anti-CD28 antibody in the presence of titrated concentrations of AZD6244 ranging from .005µM to 20µM. Flow analysis for CTLA-4 was performed on all ki67+, CD4+ T cells.

Based upon the data presented in Figures 1-5 as well as additional data not presented here, we have reached the following conclusions.

- (1) When evaluated at a population level, i.e. all CD4+ cells, AZD6244 generally functions to attenuate T cell activation by CD3/CD28 (as well as other activation signals).
- (2) However, subpopulations within the CD4+ T cell population may be more or less affected by drug (i.e. regulatory T cells appear relatively resistant to the lower doses of AZD6244)
- (3) Additionally, by focusing on specific subpopulations of cells – such as ki67+CD4+ cells (proliferating T cells) more diverse phenotypic changes are evident. Within a range of drug dosing that preserves viability of T cells, there are fewer proliferating cells at high concentrations of drug. However, those proliferating cells that are present appear to have a unique phenotype characterized by much higher expression of CTLA-4 and GITR, stable/slightly increased (rather than decreased) expression of CD25 and ICOS, and diminished expression of CD69 and PD-1.

These findings are presently being followed up with additional subset analysis of T cells stimulated in the presence of BRAFi and MEKi in vitro and in vivo.

**1b) Evaluate the effect of targeted inhibitors on activation and expansion of tumor-antigen specific transgenic T cells in vivo.**

In last years annual report, we described how MEKi may attenuate T cell signaling in vivo limiting their activation while drug is dosed. Specifically, T cells stimulated in an antigen-specific fashion in vivo have robust expansion in the presence of BRAF inhibitor, but greatly diminished in the presence of MEK inhibitor (PD901).

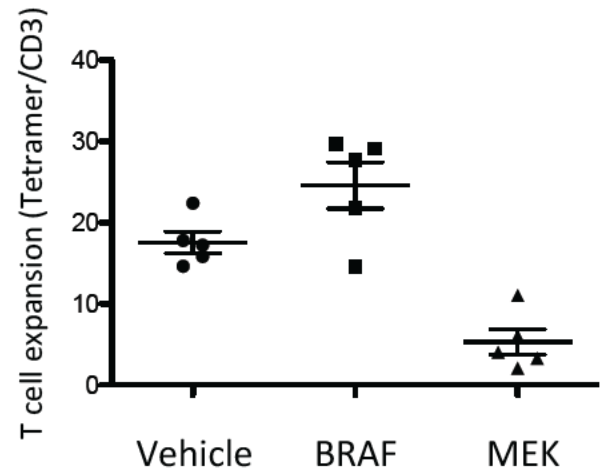


Figure 6. MEK inhibitors and BRAF inhibitors have opposing effects on T cells activated in vivo. Mice treated systemically with a vehicle control, the BRAF inhibitors PLX4720 or the MEK inhibitor PD325901 were immunized with peptide to expand antigen-specific TCR transgenic T cells. After 5 days, the expansion of antigen specific T cells was quantified by flow cytometry. Five mice were treated in each group and errors bars represent SD.

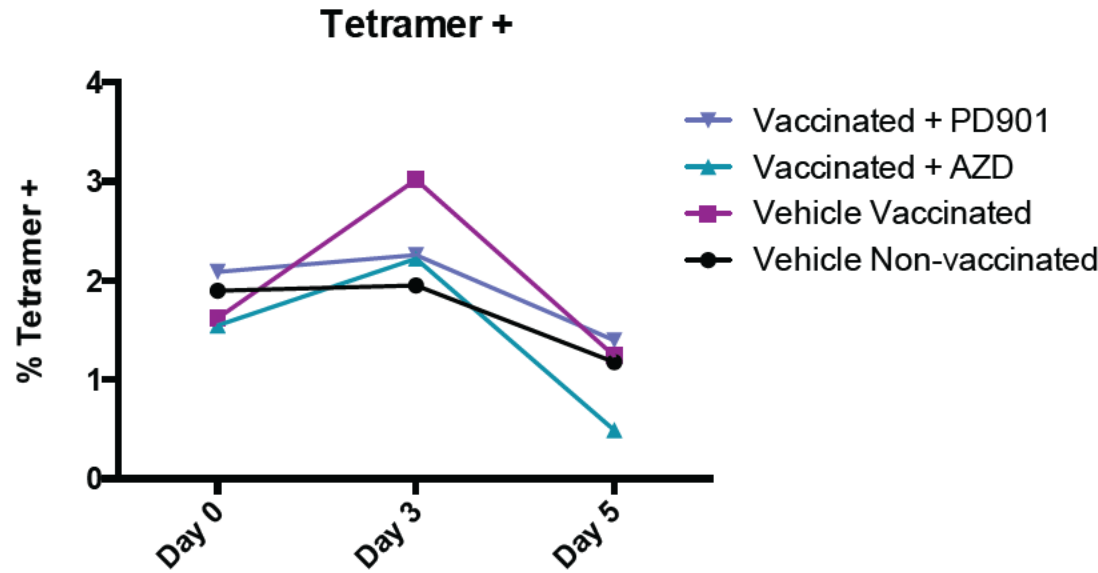


Figure 7. MEK inhibitors PD901 and AZD6244 have similar, inhibitor effects on antigen-specific T cells activated in vivo. Mice treated systemically with a vehicle control, the MEK inhibitor AZD6244 or the MEK inhibitor PD325901 were immunized with peptide to expand antigen-specific TCR transgenic T cells. After 5 days, the expansion of antigen specific T cells was quantified by flow cytometry. Five mice were treated in each group and errors bars represent SD.



Based upon these findings, we concluded that T cells activated in the presence of MEKi may have suboptimal antigen-specific activation. We hypothesized that this suboptimal activation may be explained by inhibited MAPK pathway signaling in T cells in vivo limiting their activation while drug is dosed.

To test this hypothesis, we relied on a system that we developed to test T cell MAPK pathway signaling ex vivo (Described in Figure 8).

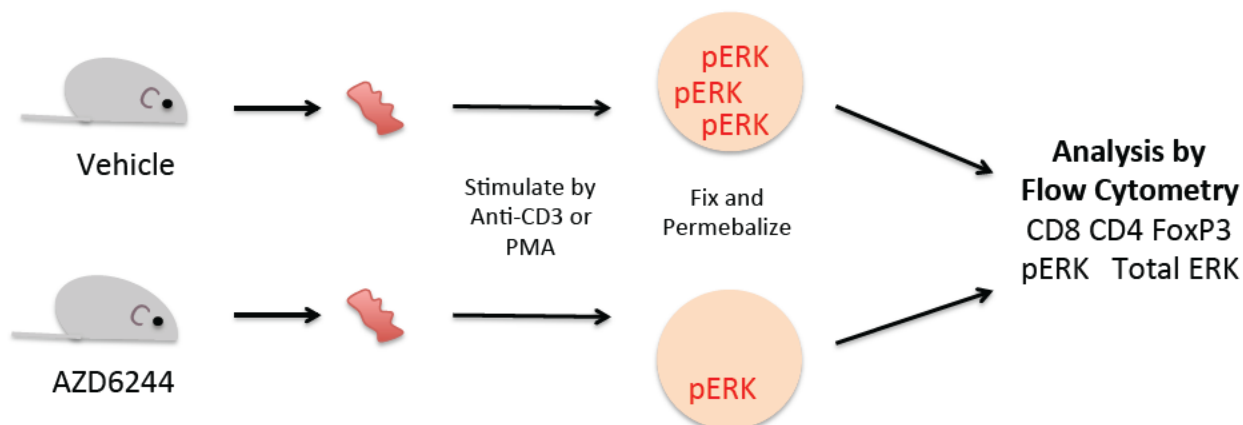


Figure 8. Mice treated systemically with the MEKi AZD6244 or vehicle control were treated for 4-5 days. Spleens were harvested and immediately ex vivo, splenocytes were stimulated and then fixed for staining with antibodies specific for pERK or ERK and T cell subset markers.

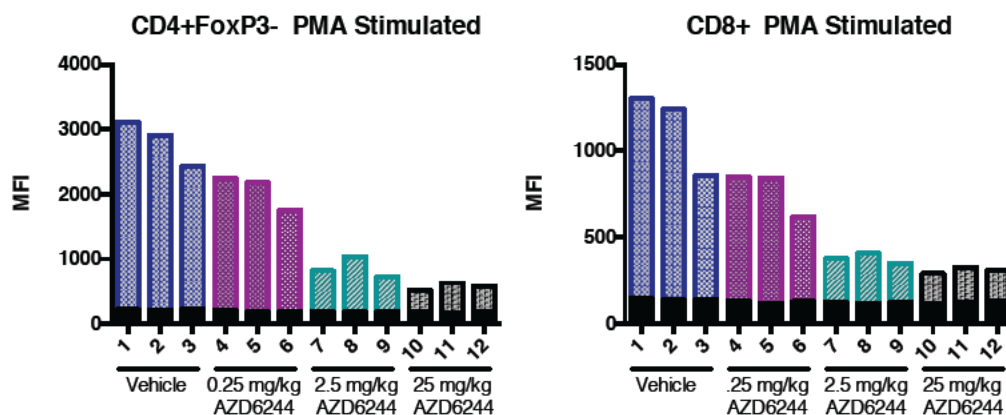


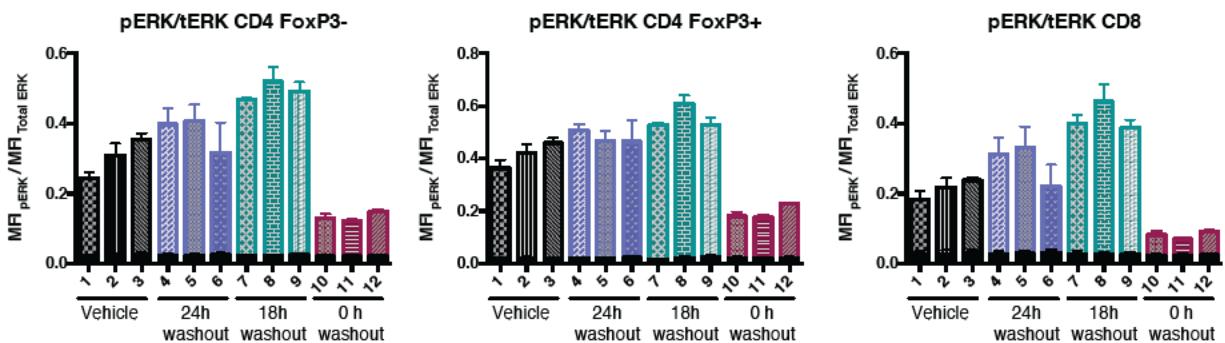
Figure 9. Mice treated systemically with the MEKi AZD6244 or vehicle control. Spleens were harvested, stimulated ex vivo and then fixed for staining with antibodies specific for pERK or ERK and T cell subset markers for CD4+ T eff cells (top left), CD8+ (top right panel) or Treg (bottom right panel).

Based on data presented in Figure 6-9, and previously discussed in the prior annual report, we reached the following conclusions:

1. MEKi appear to block activation of CD4+ (T eff), CD4+ FoxP3+ (T reg) and CD8+ T cell populations
2. This effect is dose dependent
3. This effect is true for multiple MEKi

In the past year, we have continued to exploit this system and we have begun to explore what effect(s) the MEKi may have as dosing is discontinued. We hypothesized that concurrent dosing to the MEKi at the time of generation of an antigen-specific immune response might be suboptimal and sequenced dosing or dose-interruption could be a superior, and clinically applicable approach. Essentially, the question we are aiming to address is what happens to MAPK pathway signaling in T cells after the drug is discontinued – how long until T cells recover function and are there transient or permanent changes in the characteristics of activation.

Using this approach, we have made the following novel observation that we feel could be clinically valuable in the design of combination therapies: With dosing interruption of the MEKi (and as the drug washes out) there appears to be a “rebound” effect such that T cell signaling through the MAPK is transiently enhanced, above baseline. This observation appears to be true for CD4+ T effector cells, Regulatory T cells (FoxP3+) and CD8+ T cells. As demonstrated in Figure 10, the inhibitory effects of the MEKi have worn off by 18 hours after the last dose. More notably at 18 hours, and to a less extent at 24 hours, MAPK pathway signaling is higher than baseline.



Based upon the data presented in Figure 10, we reached the conclusion that

1. A short washout period (< 1 day) after MEKi dose restores MAPK pathway signaling in T cells treated in vivo
2. For a transient period of time (~ 1 day) MAPK pathway may be capable of hyperactive signaling in T cells.

The implications of these findings are that dose interruption, and optimal timing of MEKi/immunotherapies may allow for superior combinations.

Extending these studies, we anticipate being able to better answer the question, when would the ideal time be to add checkpoint blocking antibodies and how might these combinations enhance or impair T cell activation ?

**(2) Characterize the effect of targeted inhibitors on the anti-tumor activity of checkpoint blockade (CTLA-4, PD-1) in an immunocompetent spontaneous mouse model of BRAF mutant melanoma.**

- a. Combine targeted inhibitors with CTLA-4 blockade (or PD-1 blockade) and evaluate therapeutic efficacy and immune markers in the tumor and periphery.

We have begun the process of systematically looking at immune cell subsets in mice transplanted with the WG492 cell line (derived from a melanoma cell line generated in the BRAF/PTEN transgenic mice) treated with PLX4720 or vehicle control. The WG492 cell line is described in additional detail later in this report. From this initial analysis, only minor changes have been noted thus far, but additional phenotypic analysis and comparison with MEKi drugs are underway to better characterize this system.

#### Groups:

1. Vehicle - Early
  2. Vehicle - Late
  3. PLX Treatment - Early
  4. PLX Treatment - Late
- 3 Braf-PTEN mice  
5 Braf-PTEN mice

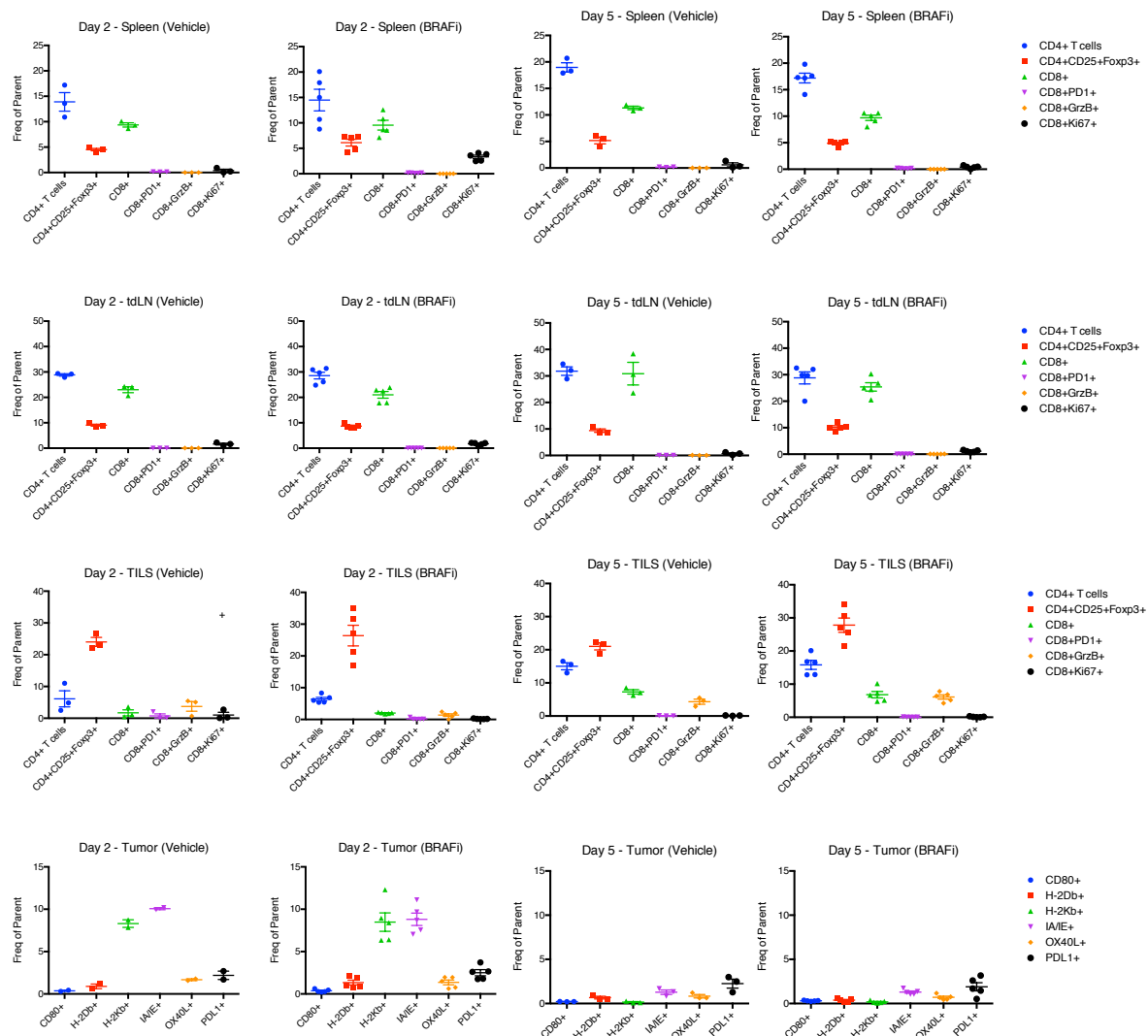
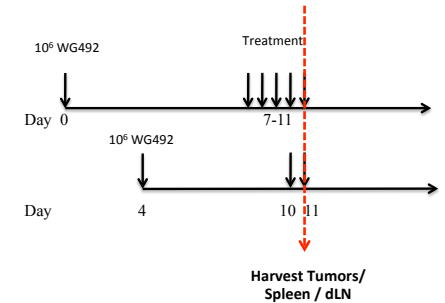


Figure 11. Mice treated systemically with the BRAFi PLX4720 or vehicle control. Spleens, draining lymph nodes and tumors were harvested. Splenocytes, cells from draining lymph nodes, and tumor infiltrating immune cells were analyzed for % of indicated populations. Tumor cells were analyzed for surface expression of CD80, MHC, OX40L and PD-L1.

**(2) Characterize the effect of targeted inhibitors on the anti-tumor activity of checkpoint blockade (CTLA-4, PD-1) in an immunocompetant spontaneous mouse model of BRAF mutant melanoma.**

**b. Compare three schedules (concurrent/sequential/alternating) combining targeted inhibitors with checkpoint blockade.**

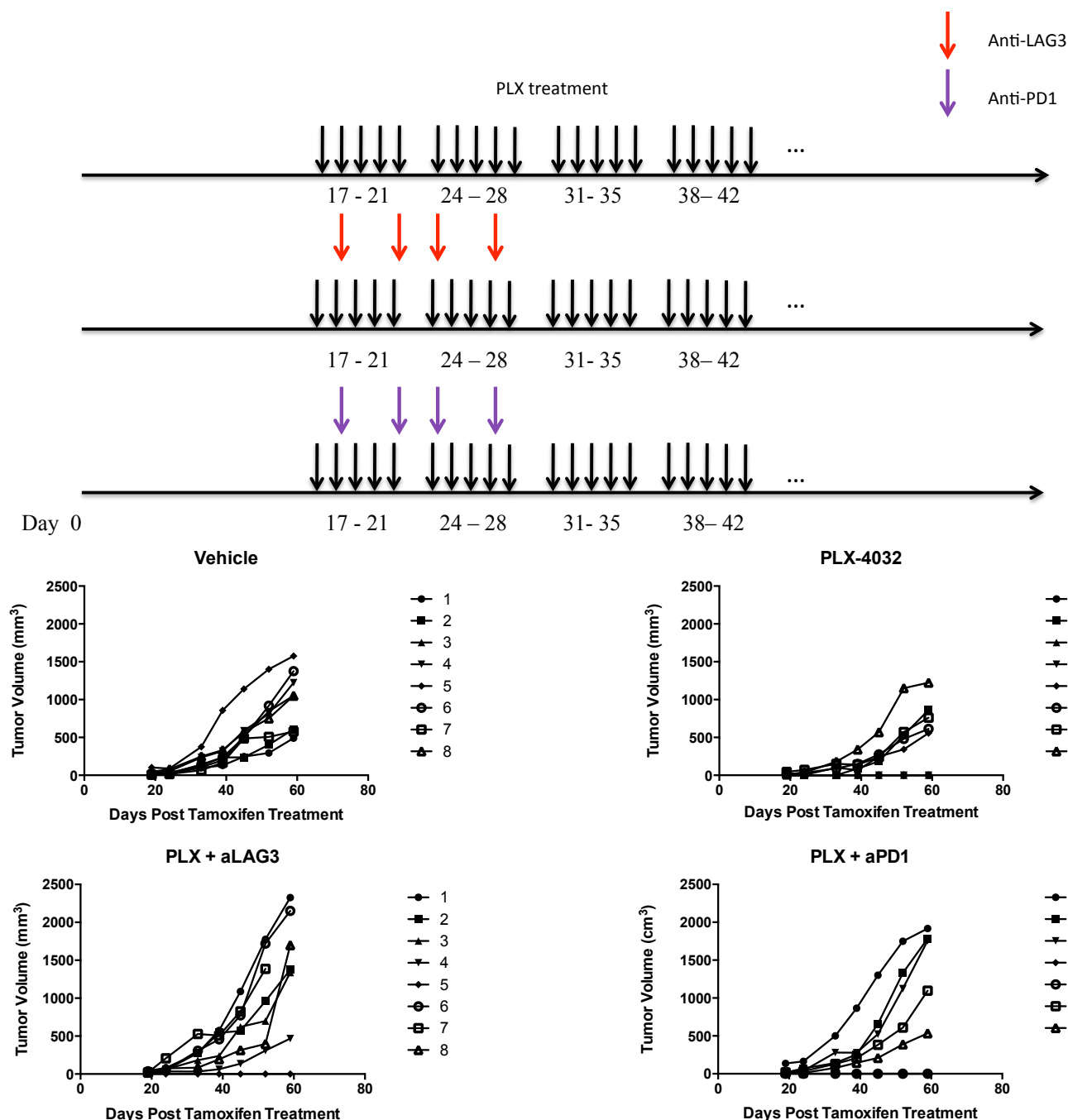


Figure 12. Mice treated systemically with the BRAFi PLX4720 or vehicle control or combination BRAFi and anti-PD01 or anti-LAG-3 antibody on a concurrent schedule. Tumor growth measured in days post Tamoxifen induction.

The data represented in Figure 12 illustrates several points that have been instructive in developing this system further. First of all, response to the BRAFi across multiple spontaneous tumors is quite variable with some tumors showing relatively high sensitivity to BRAFi (top right panel, mouse #2), whereas other appear to have very low sensitivity. This represents a significant challenge since the diversity within this group would make it challenging to reliably detect differences between BRAFi alone and in combination with immunotherapy. To understand this observation better, we have grown out individual spontaneous tumors and tested their sensitivity to BRAFi and confirmed this diverse sensitivity. To meet this challenge, we selected a single BRAF mutant tumor cell line, WG492 that (1) transplantable and grows progressively in the syngeneic, immunocompetent B6 mouse and (2) is sensitive to BRAFi as demonstrated in Figures 13-14. This approach allows us to overcome the challenge of heterogeneous sensitivity to BRAFi (as well as more general heterogeneity of the spontaneous tumors).

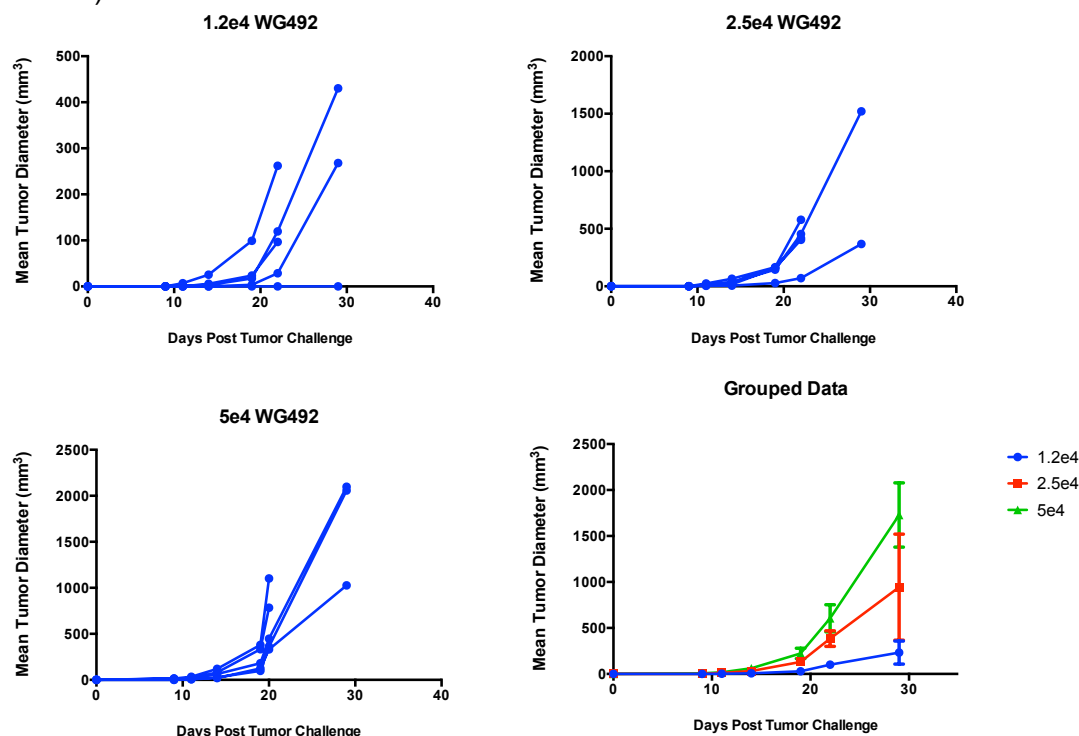


Figure 13. Immunocompetent B6 mice transplanted with titrated numbers of syngeneic WG492 BRAF mutant tumor cell line. Tumor growth measured in days post implantation.

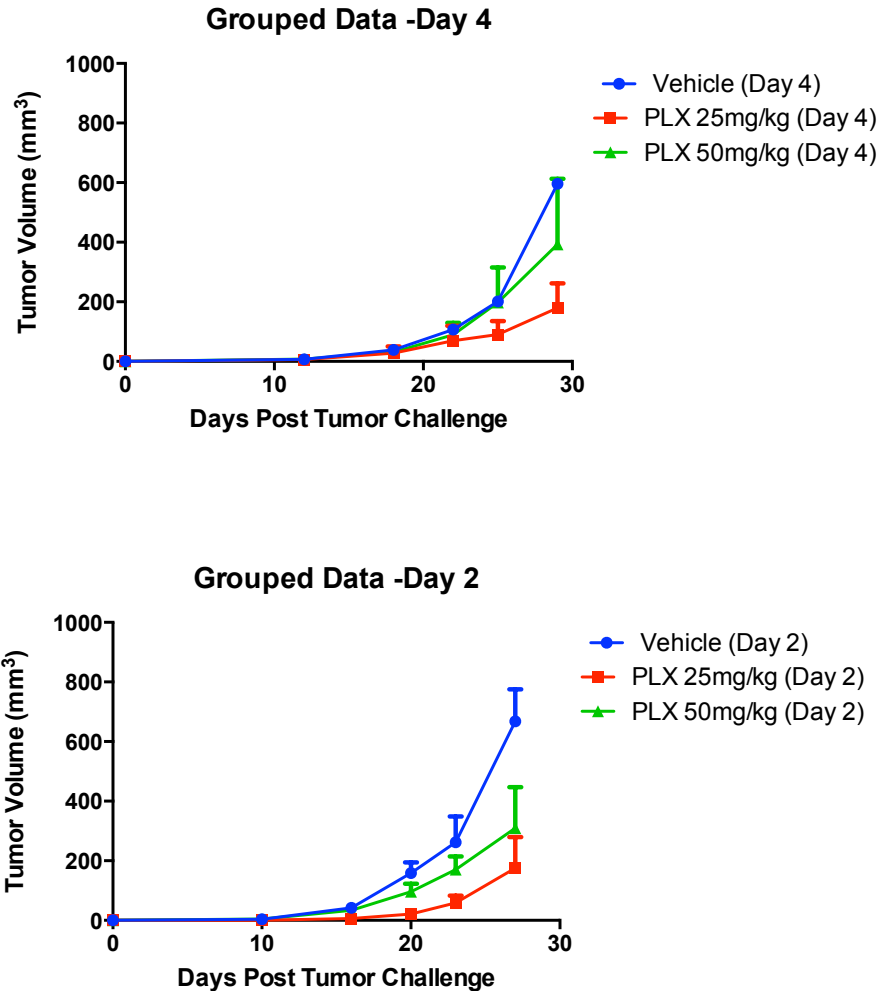


Figure 14. Immunocompetant B6 mice transplanted with syngeneic WG492 BRAF mutant tumor cell line. Tumor growth measured in days post implantation. Mice treated systemically with the BRAFi PLX4720 or vehicle control beginning on either 2 days or 4 days post implantation.

The second observation to emerge from the data in Figure 12 and similar experiments is that these spontaneous tumors appear to be relatively poorly immunogenic. It has been previously hypothesized that “spontaneous” tumor models may generate tumors that are less immunogenic since they have not acquired as many mutations (passive mutations that may be targets for an immune response) as would be acquired by tumors that develop over a longer period of time. The WG492 cell line offers us an opportunity to explore this possibility as well and we are presently working to introduce additional mutations in the WG492 cell line and test their immunogenicity/tumorigenicity.

**KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research.

- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by upregulation of a diversity of T cell activation/phenotype markers (CTLA-4, ICOS, CD25, ICOS, PD-1, CD69, ki67) in vitro
- Demonstration of inhibited T cell activation/altered phenotypes of T cell subsets in the presence of MEK inhibitors as assessed by changes in a diversity of T cell activation/phenotype markers (CTLA-4, ICOS, CD25, ICOS, PD-1, CD69, ki67) in vitro
- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by enhanced proliferation of T cells (ki67, CFSE dilution) in vitro
- Demonstration of inhibited T cell activation in the presence of MEK inhibitors as assessed by proliferation of T cells (ki67, CFSE dilution) in vitro
- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by increased ERK signaling – supporting the mechanism of paradoxical activation
- Demonstration of inhibited T cell activation in the presence of MEK inhibitors as assessed by decreased ERK signaling
- Demonstration of enhanced T cell activation in the presence of BRAF inhibitor as assessed by enhanced proliferation of T cell in vivo
- Demonstration of inhibited T cell activation in the presence of MEK inhibitors as assessed by proliferation of T cells in vivo.
- Demonstration of the duration of T cell MAPK pathway inhibition after MEK inhibitor dosing is discontinued.
- Demonstration of a transient rebound/hyperactivation of the MAPK pathway in T cells after MEK inhibitor dosing is discontinued.
- Development of a human T cell activation multiparametric flow cytometry panel
- Development and expansion of the mouse transgenic (BRAF/PTEN) colony to support mouse experiments in Years 2-3.
- Initial testing of immunotherapy (CTLA-4, PD-1 or LAG-3 blockade) and targeted inhibitor (PLX4720) combinations in murine transgenic (BRAF/PTEN) model of spontaneous melanoma and identification of potential shortcomings/strategies for improvement in the transgenic system (low immunogenicity, variable BRAFi sensitivity).
- Development and characterization of a cell line from the murine transgenic (BRAF/PTEN) model of spontaneous melanoma as a potential superior approach to testing combinations.

## REPORTABLE OUTCOMES:

The following reportable outcomes have been accomplished during this funding period.

- Manuscripts published

Callahan, et al. Paradoxical activation of T cells via augmented ERK signaling mediated by a RAF inhibitor. **Cancer Immunology Research**. 2(1): 70-9. 2014.

- Abstracts and presentations

None during this reporting period

- Employment or research opportunities applied for and/or received based on experience/training supported by this award

Margaret Callahan, MD, PhD was appointed to a faculty position (Assistant Attending) at the Memorial Sloan-Kettering Cancer Center as a result of experience/training supported by this award.

Dr. Callahan and Dr. Merghoub have applied (as co-investigators) for a SU2C team science grant to study the effect of TKIs used in the treatment of NSCLC on immune cells, based upon experimental approaches and techniques developed based upon this award.

Dr. Callahan and Dr. Merghoub have applied for and received (as co-investigators) internal (MSKCC) funding to extend the work on MEKi as immune modulators based upon experimental approaches and techniques developed based upon this award.



## CONCLUSION:

In these studies, we have characterized the effects of BRAFi and MEKi on T cell activation – highlighting two areas that may be clinically relevant to the combination of targeted inhibitors and immunotherapy (1) the paradoxical T cell activation by BRAF inhibitors that results in increased T cells upregulation of activation markers, cytokines and proliferation (2) the rebound hyperactivation of T cells after discontinuation of MEK inhibitor treatment. These findings represent mechanisms that may be exploited to maximize the clinical benefit of combination therapies and will be applied to guiding the design of combination therapies in mouse models.

We have also laid the groundwork for mouse models to test these combinations and anticipate that in the coming year we will be well equipped to generate data on concurrent, sequenced and intermittent dosing combination regimens.

Observations thus far have several implications for the development of combination therapies in the clinic and have generated new questions to be explored at the bench and in the clinic including:

1. Can paradoxical activation be exploited to enhance the anti-tumor T cell activity of immunotherapies ?
2. Can paradoxical activation of T cells be exploited in other clinical scenarios (i.e. vaccines) where robust T cell activation is desired ?
3. Will paradoxical activation be a liability for combination therapy and how would this impact the toxicity profile for combination therapies ?
4. How will the T cell effects of MEK inhibitors effect the clinical activity of combination therapy in the short term ? in the long term (T cell memory) ?
5. Will the rebound hyperactivation of T cells after discontinuation of MEK inhibition present an opportunity for combination with checkpoint blockade or vaccine therapy ?

## REFERENCES:

1. Aris M, Barrio MM. Combining immunotherapy with oncogene-targeted therapy: a new road for melanoma treatment. *Frontiers in immunology* 2015;6:46.
2. Tomei S, Bedognetti D, De Giorgi V, et al. The immune-related role of BRAF in melanoma. *Molecular oncology* 2015;9:93-104.
3. Gargett T, Fraser CK, Dotti G, Yvon ES, Brown MP. BRAF and MEK inhibition variably affect GD2-specific chimeric antigen receptor (CAR) T-cell function in vitro. *J Immunother* 2015;38:12-23.
4. Wargo JA, Cooper ZA, Flaherty KT. Universes collide: combining immunotherapy with targeted therapy for cancer. *Cancer discovery* 2014;4:1377-86.
5. Vella LJ, Pasam A, Dimopoulos N, et al. MEK inhibition, alone or in combination with BRAF inhibition, affects multiple functions of isolated normal human lymphocytes and dendritic cells. *Cancer immunology research* 2014;2:351-60.
6. Smith MP, Sanchez-Laorden B, O'Brien K, et al. The immune microenvironment confers resistance to MAPK pathway inhibitors through macrophage-derived TNFalpha. *Cancer discovery* 2014;4:1214-29.
7. Jazirehi AR, Nazarian R, Torres-Collado AX, Economou JS. Aberrant apoptotic machinery confers melanoma dual resistance to BRAF(V600E) inhibitor and immune effector cells: immunosensitization by a histone deacetylase inhibitor. *American journal of clinical and experimental immunology* 2014;3:43-56.
8. Hu-Lieskovan S, Robert L, Homet Moreno B, Ribas A. Combining targeted therapy with immunotherapy in BRAF-mutant melanoma: promise and challenges. *J Clin Oncol* 2014;32:2248-54.
9. Ferrari de Andrade L, Ngiow SF, Stannard K, et al. Natural killer cells are essential for the ability of BRAF inhibitors to control BRAFV600E-mutant metastatic melanoma. *Cancer Res* 2014;74:7298-308.
10. Callahan MK, Masters G, Pratilas CA, et al. Paradoxical activation of T cells via augmented ERK signaling mediated by a RAF inhibitor. *Cancer immunology research* 2014;2:70-9.